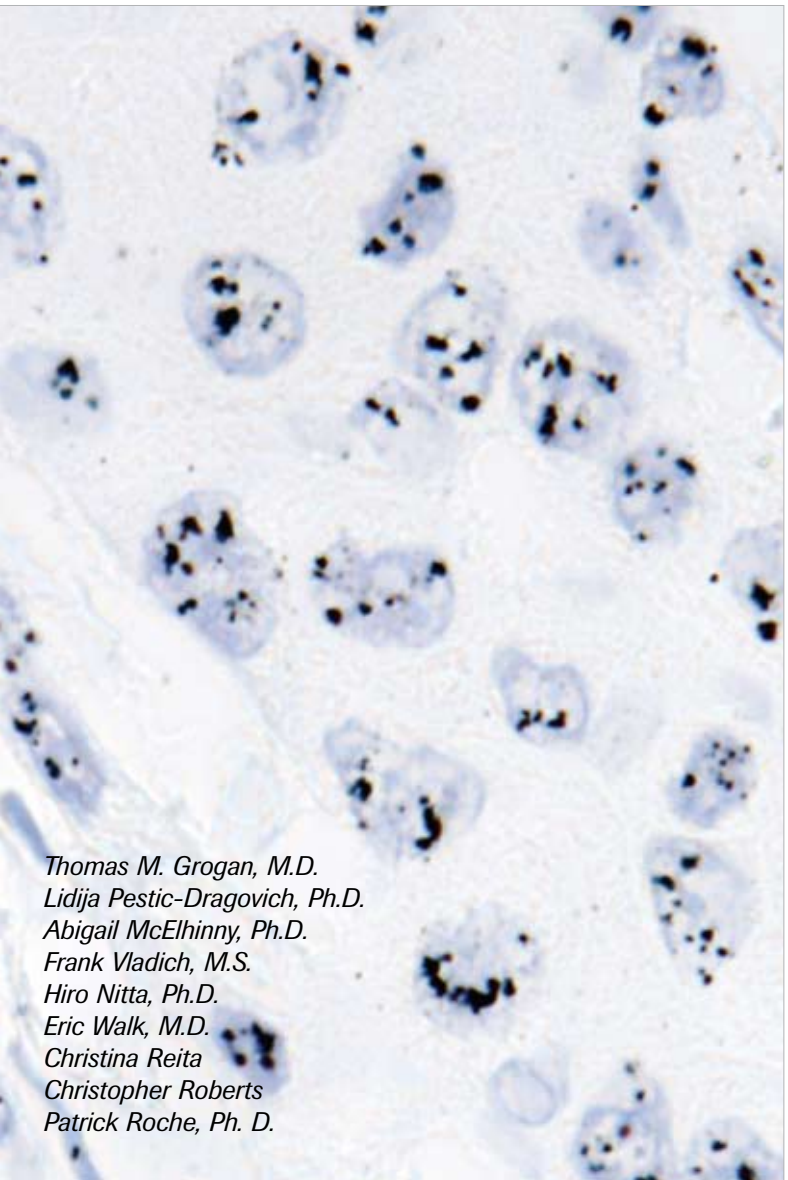
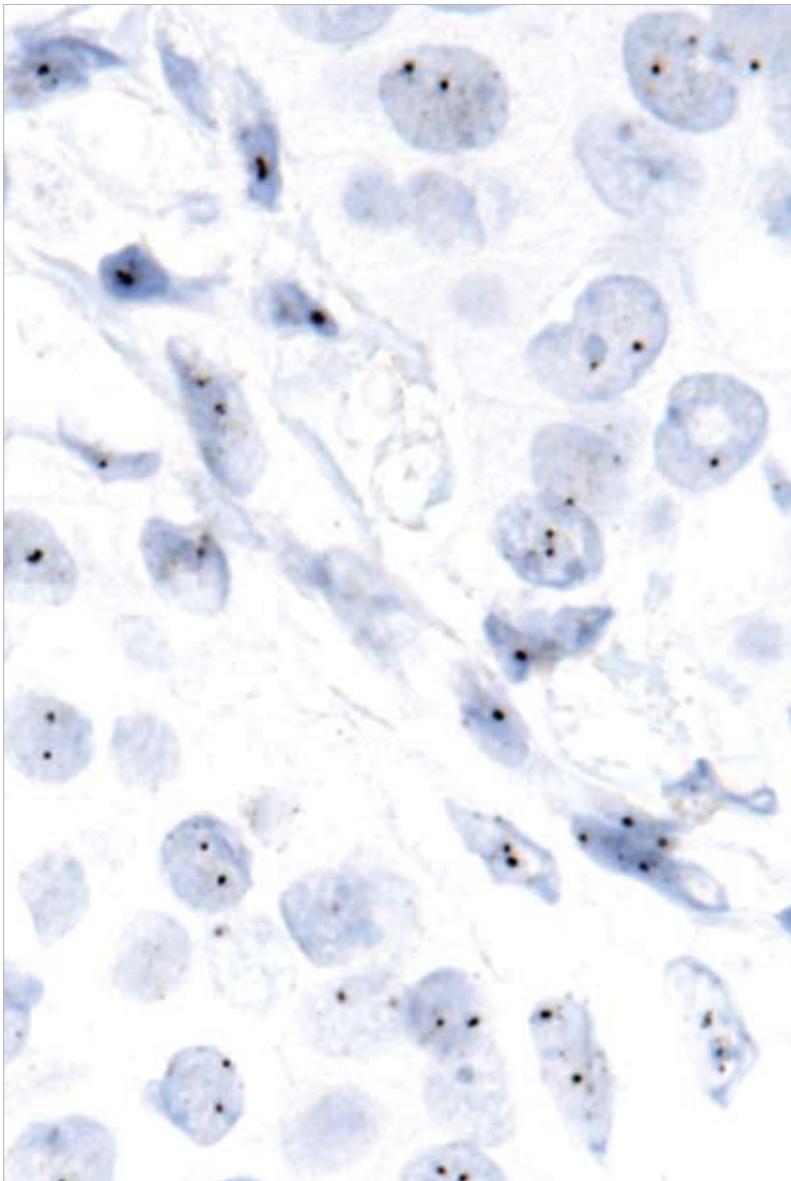
Interpretation Guide
INFORM™ HER2

DNA Probe Staining of Breast Carcinoma





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INTRODUCTION 1
- General Description of INFORM™ *HER2* DNA Probe 1
- Purpose of Interpretive Guide 1

IDENTIFICATION OF APPROPRIATE STAINING PATTERN 2
- *HER2* Gene Status 2
- Signal Visualization and Enumeration of SISH Staining 4
- Target Area 4
- Slide Adequacy 5
- Selecting Carcinoma Nuclei for Enumeration 6
- Additional Observations 6

DETERMINING *HER2* GENE STATUS 8
- Method 1: Semi-Quantitiative Method 9
- Method 2: Quantitative Method 10
- Method 2A: Additional Quantitation 11

EXAMPLES OF *HER2* AND CHR17 STAINING PATTERNS IN CLINICAL CASES 12

QUALITY CONTROL FOR SISH STAINING 14
- Positive and Negative Controls for SISH Staining 14
- *HER2* 3-in-1 Xenograft Control Slides 14

PRE-ANALYTICAL CONSIDERATIONS AND OPTIMIZATION OF THE ASSAY 16
- Fixation 16
- Sample Thickness 16
- Other Considerations 16

TROUBLESHOOTING 18

REFERENCES 21

APPENDIX A 22
- Score Sheets 22

Introduction

General Description of INFORM™ *HER2* DNA Probe

Ventana Medical Systems' INFORM *HER2* DNA Probe is designed to quantitatively detect amplification of the *HER2* gene via chromogenic silver *in situ* hybridization (SISH) in formalin-fixed, paraffin-embedded human breast cancer tissue specimens, following staining on Ventana automated slide stainers using light microscopy. The INFORM HER2 DNA Probe is indicated as an aid in the assessment of patients for whom Herceptin® (trastuzumab) treatment is being considered. Results from the INFORM *HER2* DNA Probe are intended for use as an adjunct to existing clinical and pathologic information currently used for estimating prognosis in patients with invasive breast cancer.

A qualified reader experienced in the microscopic interpretation of breast carcinoma specimens, ISH procedures and the recognition of single and amplified *HER2* copies (which may require microscopic examination using objectives as high as 40X to 60X) must evaluate controls before interpreting results.

Purpose of Interpretive Guide

The following cases illustrate the variety of staining patterns that may be present in breast biopsies when stained with INFORM *HER2* DNA Probe and Chromosome 17 Probe. The photomicrographs allow new users to become familiar with the spectrum of staining patterns including single-copy staining of *HER2* and Chromosome 17, amplified gene copies and clusters of *HER2* staining, as well as artifact staining that they may encounter. Additionally, the images allow users to become familiar with determination of slide adequacy, enumeration methods, and troubleshooting of the assay. The intent is to provide pathologists with a tool to facilitate interpretation of INFORM *HER2* DNA and Chromosome 17 Probes' staining patterns. Any staining performed in the end user's lab should be interpreted within the context of the controls run with the clinical cases at the time of evaluation. See the package insert provided with these products for further information, and the Quality Control section of this guide.

The images contained in this interpretive guide were obtained using the INFORM *HER2* DNA and Chromosome 17 Probes in the assay developed and validated on Ventana automated slide stainers under the direction of Ventana Medical Systems, Inc.

Identification of Appropriate Staining Pattern

HER2 Gene Status

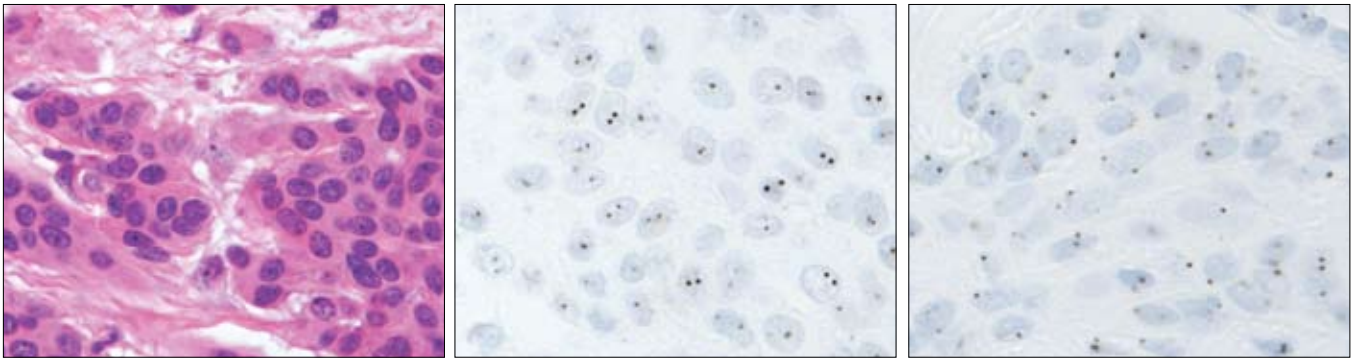
The *HER2* gene is located on human Chromosome 17 and encodes the HER2 protein. Amplification of the *HER2* gene occurs in approximately 15 to 25 percent of breast cancers, and is associated with aggressive tumor behavior.[1-5] In many clinical studies, amplification and/or overexpression of *HER2* has been shown to be associated with a poor clinical outcome for women with invasive breast cancer and correlated with several negative prognostic variables, including estrogen receptor (ER) negative status, high S-phase fraction, positive nodal status, mutated p53, and high nuclear grade.[6,7]

HER2 gene status is reported as a function of the ratio of the average number of copies of the *HER2* gene to the average number of copies of Chromosome 17 (Chr17) per cell in an invasive breast carcinoma. *HER2* gene status is classified using the following guidelines.[22] A sample is negative for *HER2* gene amplification if the *HER2*/Chr17 ratio is less than 1.8 (Figure 1, Case 1). A sample is equivocal for *HER2* gene amplification if the *HER2*/Chr17 ratio is either equal to or falls between 1.8 and 2.2 (Figure 1, Case 2). Finally, if the *HER2*/Chr17 ratio is greater than 2.2, a sample is positive for *HER2* gene amplification (Figure 1, Case 3). These classifications are summarized in Table 1.

Ventana has designed this assay so that the INFORM *HER2* DNA Probe is detectable on one slide and the INFORM Chromosome 17 Probe is detectable on a matched slide. The probes are visualized using silver *in situ* hybridization (SISH) and appear as discrete black dots in the nuclei of normal cells (serving as internal positive controls for staining) and in invasive carcinoma. This strategy allows *HER2* gene status to be determined in the context of its chromosomal state, using standard light microscopy with 20X, 40X, and/or 60x objectives. Ventana has developed two methods for enumerating *HER2* gene status: a semi-quantitative method (Method 1) and a quantitative method (Method 2). These methods are discussed in greater detail in the Determining *HER2* Gene Status Section.

Figure 1. *HER2* Gene Status in Representative Cases

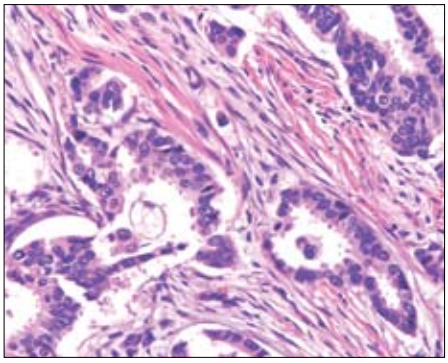
Case 1 is negative for *HER2* gene amplification



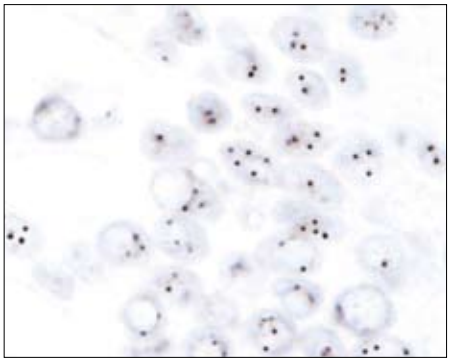
Case 1: H&E 20X | *HER2* 60X | Chr17 60X

Figure 1. (cont.) *HER2* Gene Status in Representative Cases

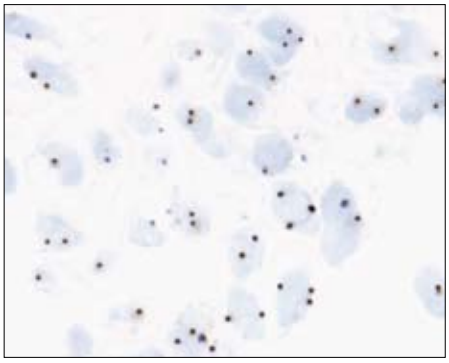
Case 2 is equivocal for *HER2* gene amplification



Case 2: H&E 20X

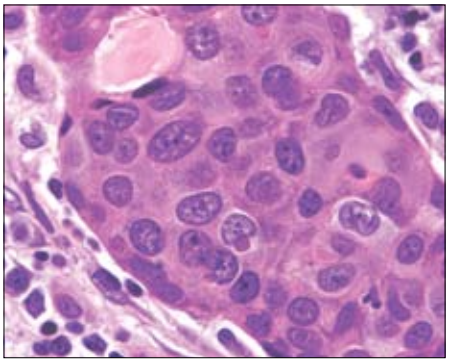


HER2 60X

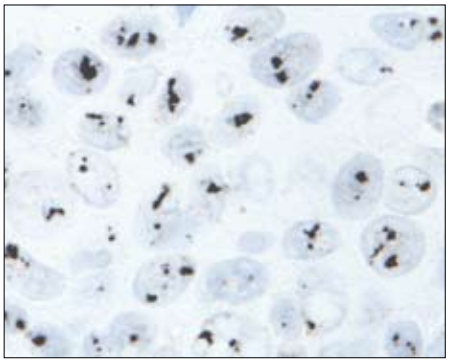


Chr17 60X

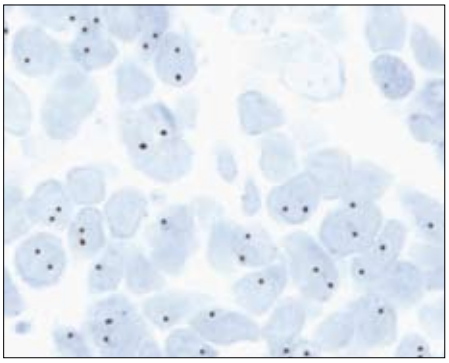
Case 3 is positive for *HER2* gene amplification



Case 3: H&E 20X



HER2 60X



Chr17 60X

Table 1. *HER2* Testing Reported Results

Average *HER2*/ Chr17 ratio	**Reported Result**
HER2/Chr17 < 1.8	Negative for *HER2* gene amplification
1.8 ≤ *HER2*/Chr17 ≤ 2.2	Equivocal for *HER2* gene amplification
HER2/Chr17 > 2.2	Positive for *HER2* gene amplification

Table 2. Signal Enumeration

	Single Copy Count as 1.
	Multiple Copies Count as 2.
	Small Clusters Count as 8. One small cluster (6) and 2 discrete dots are present.
	Large Cluster Count as 16. One large cluster (12) and 4 discrete dots are present.

Signal Visualization and Enumeration of SISH Staining

SISH signals are visualized as single copies, multiple copies, and clusters (Table 2).

Single Copy

A discrete dot is counted as a single copy of *HER2* or Chr17. Discrete single dots visualized in the internal positive control nuclei represent the size of a single copy in invasive carcinoma cells (See Figure 2, Case 4). It is important to note that the discrete dots representing a single copy of either *HER2* or Chr17 may appear larger in some patient samples than in others. Because of this, it is important to use the single dots visualized in the internal positive control nuclei adjacent to the target area as a reference. The internal positive control nuclei occur within normal adjacent stromal cells (e.g. fibroblasts, fibrocytes, and endothelials) and leukocytes (e.g. lymphocytes and macrophages) (Figure 3).

Multiple Copies

As described above, discrete single dots visualized in the internal positive control nuclei represent the size of a single copy in invasive carcinoma cells. The size of these single dots is used as a reference to determine the relative number of amplified copies in the cancer nuclei. Figure 2, Case 5 shows a number of nuclei in which multiple discrete copies are visible.

Clusters

In some nuclei, clusters of dots representing many copies of *HER2* gene are apparent. A small cluster of multiple signals is counted as six signals, and a large cluster as 12 signals. It is possible for a single nucleus to have multiple small clusters, multiple large clusters, or a combination of large and small clusters. Figure 2, Case 6 shows a number of nuclei in which clusters are visible, both small and large.

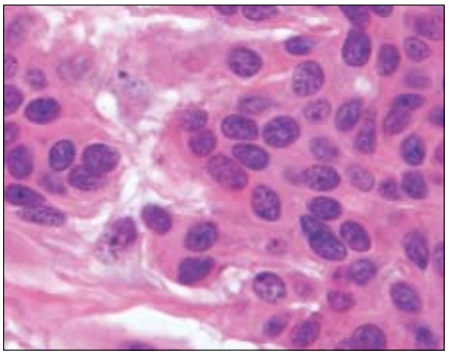
Target Area

It is important that the reader identify acceptable target areas for signal visualization and enumeration within the sample. An acceptable target area must be located within the invasive breast carcinoma; carcinoma *in situ* should not be scored.

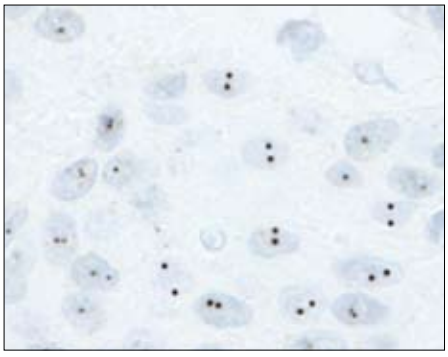
Once located, the target area must satisfy two criteria to be deemed adequate for enumeration. This is discussed thoroughly in Slide Adequacy (D).

Figure 2. Signal Visualization

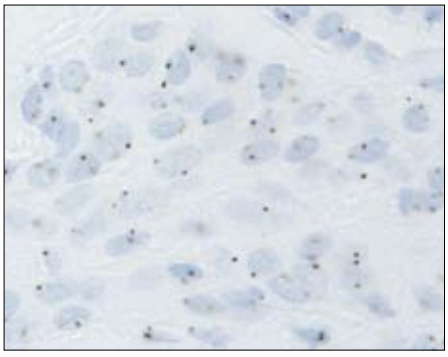
Single Copy



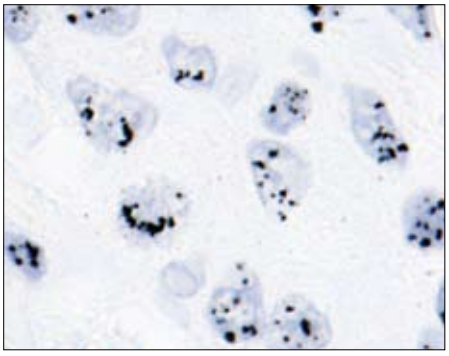
Case 4: H&E 60X



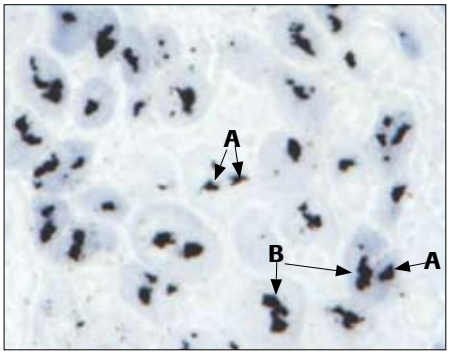
HER2 60X



Chr17 60X



Case 5: H&E 60X
Multiple copies and small clusters



Case 6: *HER2* 60X
Small (A) and large (B) clusters

Slide Adequacy

Before enumerating a *HER2* or Chr17 stained slide, it is critical that the reader determine whether the target area is adequately stained. The target area must satisfy two criteria to be deemed adequate for enumeration. If the slide does not meet these criteria, it cannot be enumerated. The user should refer to the troubleshooting section and evaluate the appropriate conditions for re-staining the slide.

Internal Positive Control Staining must be present

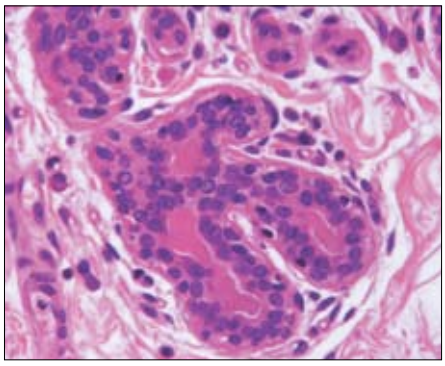
Normal *HER2* or Chr17 signals (one to two copies per cell that are visible as distinct "Single Copy" staining) act as internal positive controls and should be visible using 20X, 40X, or 60X objectives. This distinct nuclear staining may be located in various non-neoplastic cells including: stromal fibroblasts, endothelial cells, lymphocytes, and benign breast epithelial cells (Figure 3). Due to truncation artifacts in the plane of sectioning, it usually is not possible to visualize single *HER2* gene or Chr17 copy number in all cells on the slide, nor in all regions of the tissue. However, accurate enumeration requires that single-copy SISH signals are visible in normal cells within and/or adjacent to the target area (See Figures 1-3). Figure 4 shows an example of a slide that would be considered inadequate due to a lack of internal positive control staining in or adjacent to the target area.

SISH staining within the invasive breast carcinoma cells in the target area must be enumerable

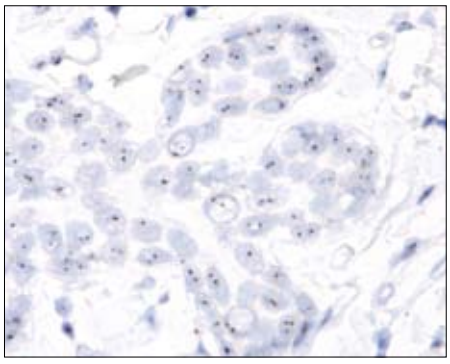
Using 20X, 40X, and/or 60X objectives, the invasive breast carcinoma in the target area must exhibit an enumerable field of *HER2* or Chr17 SISH signals. Due to truncation in the plane of sectioning, it is likely that not every carcinoma nucleus will contain SISH signals. However, it is important that the target area contains an acceptable region that is enumerable. If a particular target area is deemed too weak to enumerate, it is often possible to enumerate a different target area on the same slide. If acceptable SISH staining within any of the target areas is not present, then the slide is considered inadequate and cannot be enumerated. Figure 5 is an example of a target area that would be considered inadequate.

Figure 3. Normal Staining in Internal Positive Control Nuclei

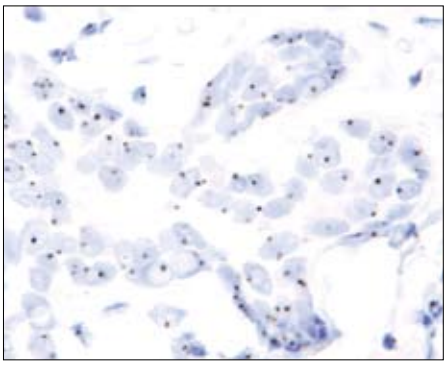
Case 2 is equivocal for *HER2* gene amplification



Case 7: H&E 60X

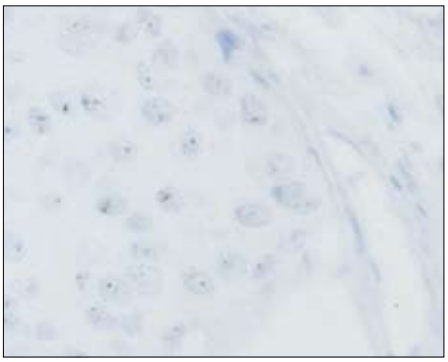


HER2 60X



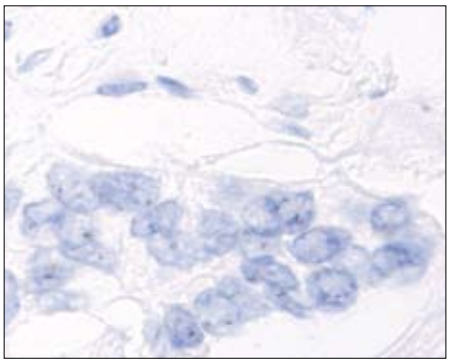
Chr17 60X

Figure 4. Inadequate Due to a Lack of Internal Positive Control Staining in or Adjacent to the Target Area



Case 8: *HER2* 60X

Figure 5. Inadequate Due to Weak SISH Staining Within the Target Area



Case 9: *HER2* 60X

Selecting Carcinoma Nuclei for Enumeration

Once a target area that exhibits adequate SISH staining is located, only representative carcinoma nuclei should be enumerated. Signal enumeration should not be performed in areas that contain weak SISH signals (i.e., no internal control cell staining present), compressed or overlapping nuclei, or necrosis. Additionally, signal enumeration should not be performed in nuclei that are not representative of the general population of invasive carcinoma nuclei in the target area. Abnormal, giant nuclei (2-fold or greater the size of other carcinoma nuclei) and small nuclei (about half the size of the other carcinoma nuclei) should not be enumerated. Finally, in target areas that are genetically heterogeneous for *HER2* copy number, count only nuclei that are representative of the population of invasive carcinoma nuclei with the highest average number of signals. Heterogeneity is discussed in greater detail in Additional Observations.

Additional Observations

Other observations regarding the *HER2* or Chr17 SISH staining may be clinically significant and should be noted as comments on the pathologist's report.

Heterogeneity

Occasionally, tissue samples may contain breast carcinoma nuclei that are genetically heterogeneous for *HER2* copy number. In these cases, there can be a mixture of nuclei that are amplified and not amplified and/or a mixture of nuclei containing various numbers of copies of *HER2*. This may be observed among carcinoma cells within the same target area, or between two different target areas within the tissue. It is recommended that the nuclei with the higher numbers of *HER2* copy number be chosen for enumeration, but that the heterogeneity in relative copy number be noted on the patient's report.

Polysomy

Polysomy is a condition in which the carcinoma nuclei contain at least one more chromosome than normal (i.e. the average number of Chromosome 17 copies is not diploid). In these nuclei, there may be three or more copies of the chromosome (Figure 6, Case 11 and Case 12). *Note: This is not to be confused with diploid, dividing nuclei present throughout the tissue that contain 3-4 copies of Chromosome 17).*

Monosomy

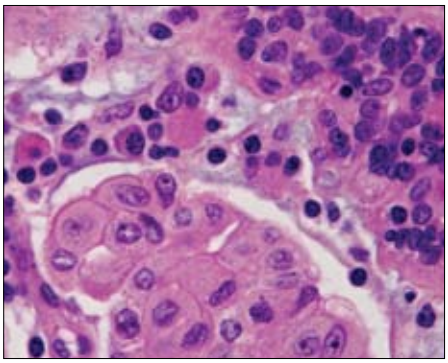
Monosomy is a condition in which the carcinoma nuclei contain only one copy of Chromosome 17.

Monoallelic Deletion

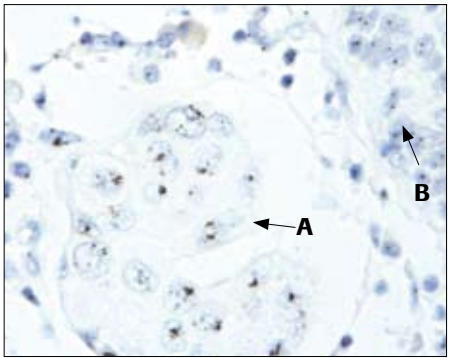
Monoallelic Deletion is the deletion of the *HER2* gene from Chromosome 17 (Figure 6, Case 12).

Figure 6. Additional Observations Regarding *HER2* or Chr17 Staining

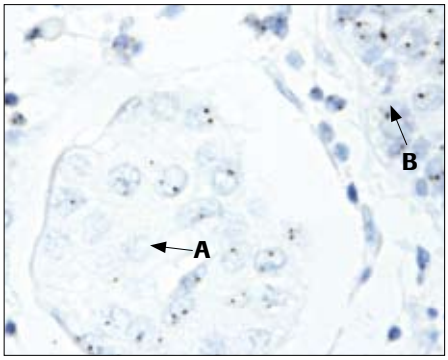
The *HER2* image for Case 10 contains both invasive carcinoma (A) and atypical ductal hyperplasia (B). If the hyperplasia were enumerated, the case would be negative. It is important to only enumerate invasive carcinoma and use the H&E slide as a guide.



Case 10: H&E 60X

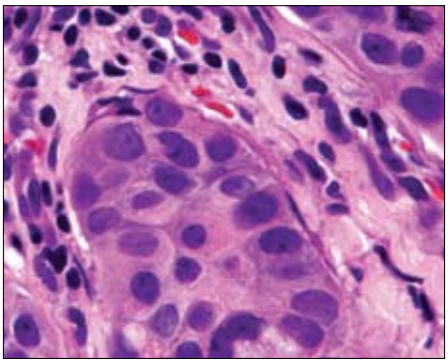


HER2 60X

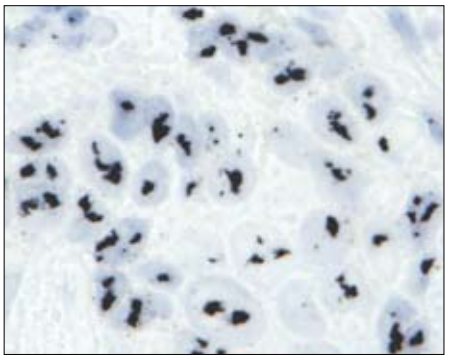


Chr17 60X

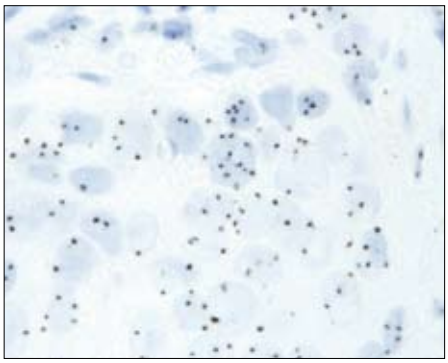
Case 11 is positive for *HER2* gene amplification but note coincidental Polysomy Chromosome 17.



Case 11: *HER2* 60X

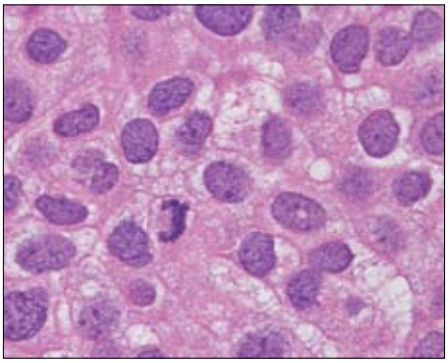


Case 9: *HER2* 60X

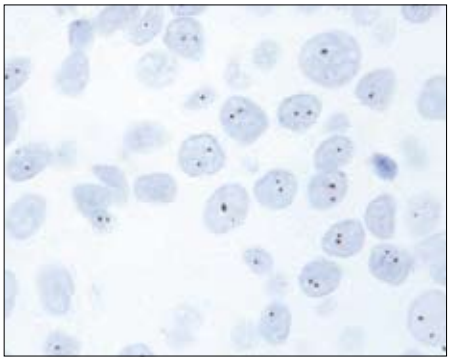


Chr17 60X

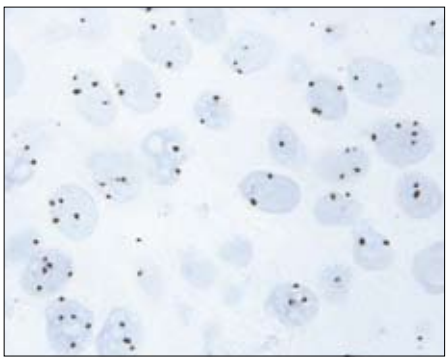
HER2 copy number for Case 12 is approximately 2, but note that Chromosome 17 is polysomic. Thus, Chromosome 17 was duplicated, but there was also a genetic loss of the *HER2* allele. The case report should note Monoallelic Deletion for Case 12.



Case 12: H&E 60X



HER2 60X



Chr17 60X

Determining HER2 Gene Status

In a study[23] that analyzed fluorescence *in situ* hybridization (FISH) testing in clinical laboratories, the frequency of *HER2*/Chr17 ratios in invasive breast carcinoma was determined to have the distribution shown in Figure 7. To develop a scoring algorithm that fits within the workflow of a pathology laboratory and generates results that are reproducible between readers, Ventana has developed a two-part approach (Figure 8).

Figure 7. Frequency Distribution of *HER2*/Chr17 Ratios by FISH

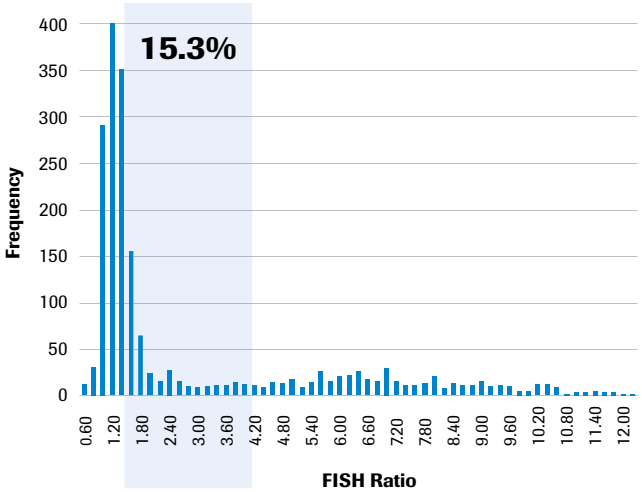
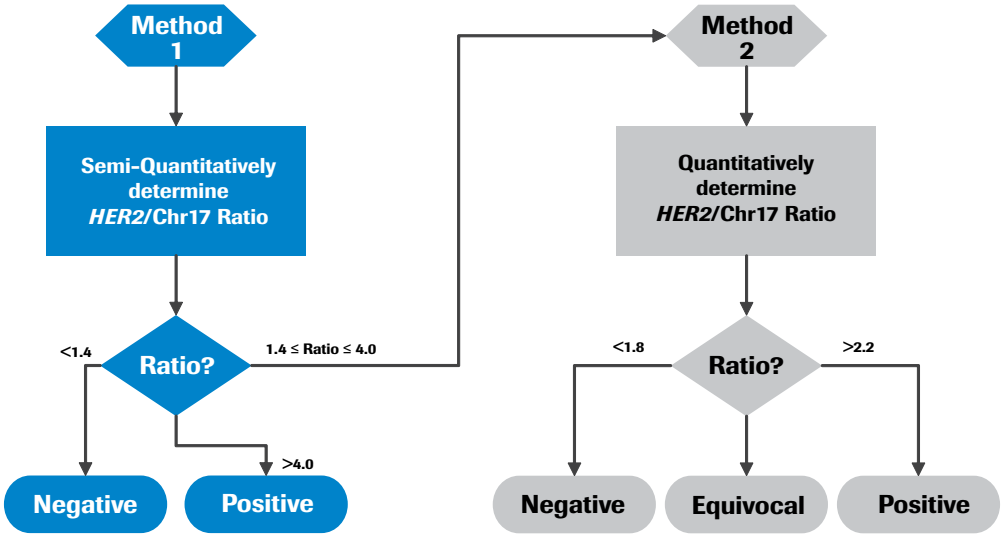


Figure 8. Overview of *HER2* SISH Scoring Algorithm



Method 1 allows the reader to rapidly and semi-quantitatively determine *HER2* gene status for approximately 85% of all cases, which are either clearly negative or positive for *HER2* gene amplification (i.e., cases with a *HER2*/Chr17 ratio less than 1.4 or greater than 4.0). This is done by analyzing the overall staining patterns of *HER2* and Chromosome 17 within a target area.

For the minority of cases in which the *HER2*/Chr17 ratio falls within the range of 1.4 to 4.0, the reader must proceed to Method 2, a quantitative method. This is recommended to ensure accuracy in determining *HER2* gene status. As cited above[23], approximately 15% of breast carcinoma cases will fall within the range of 1.4 and 4.0.

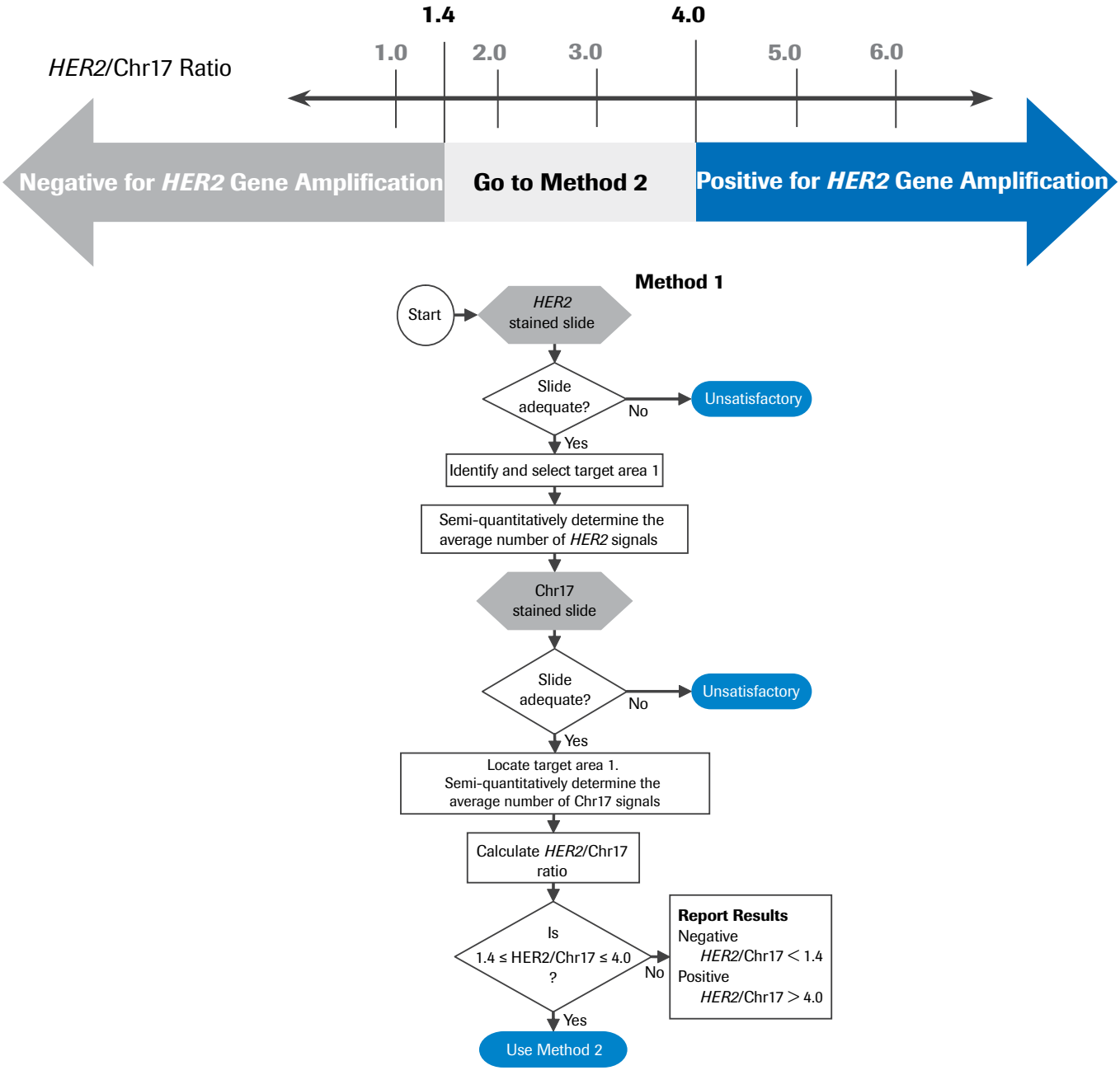
Alternately, a reader may choose to skip Method 1 and proceed directly to Method 2, the quantitative method, for enumeration of the *HER2*/Chr17 ratio.

Method 1: Semi-Quantitative Method

Using Method 1, the reader semi-quantitatively estimates the average number of *HER2* and Chr17 signals in the target area and records the *HER2*/Chr17 ratio.

Utilizing stained serial slides that meet the criteria for Slide Adequacy, locate the same target area on each slide of *HER2* and Chr17 and determine, semi-quantitatively, the average number of signals in the Target Area according to Table 2—Signal Enumeration. This is performed by analyzing the overall staining pattern for *HER2* and Chr17 within the target area. Calculate the *HER2*/Chr17 ratio by dividing the average number of *HER2* signals by the average number of Chr17 signals. Cases with a *HER2*/Chr17 ratio less than 1.4 are negative for *HER2* gene amplification while cases with a *HER2*/Chr17 ratio more than 4.0 are positive for *HER2* gene amplification and can be reported as such. Cases with a *HER2*/Chr17 ratio that is either equal to or falls between 1.4 and 4.0 must be enumerated using Method 2 to ensure accuracy (Figure 9).

Figure 9. Method 1 Scoring Algorithm

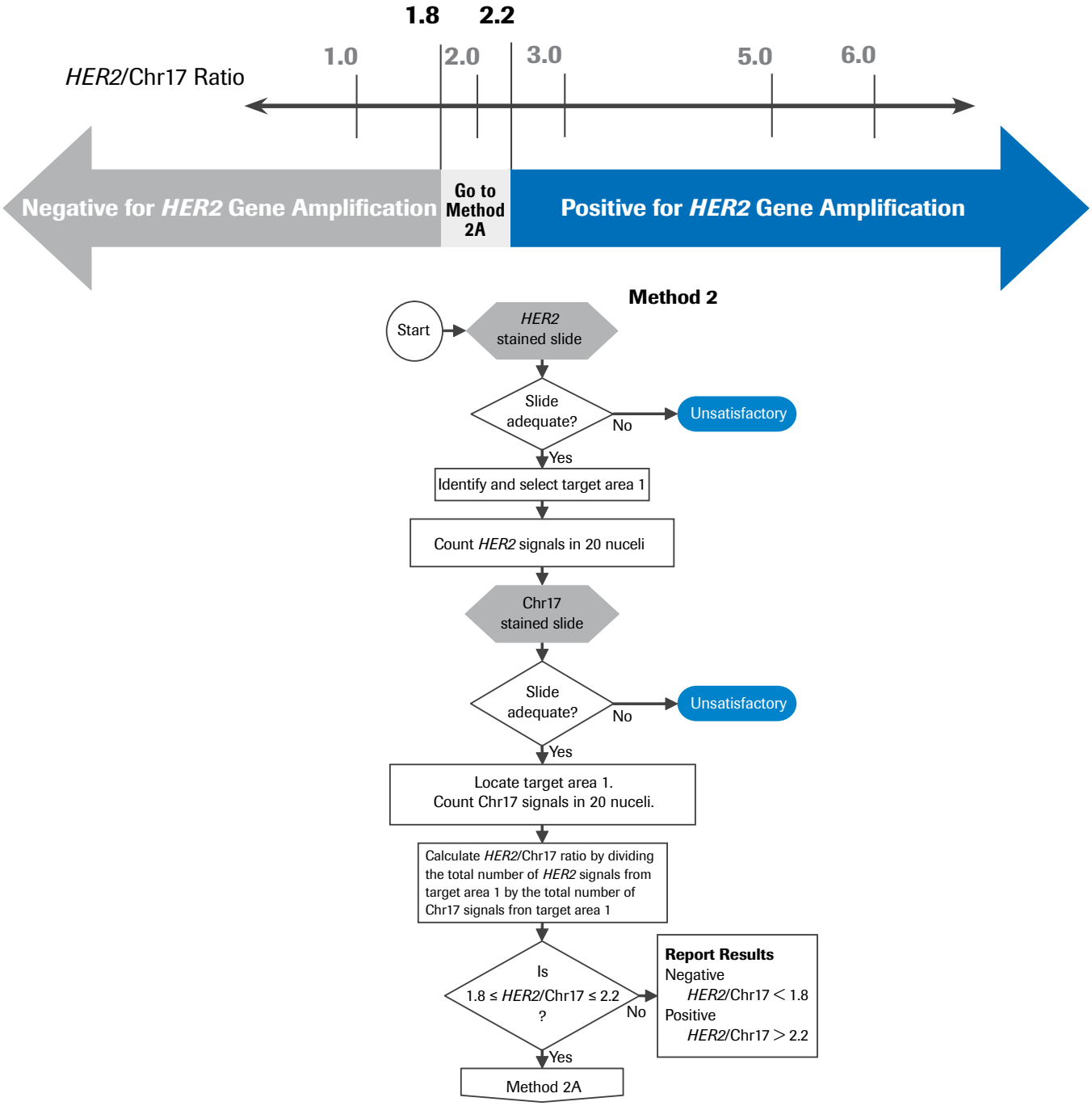


Method 2: Quantitative Method

Using Method 2, the reader records the quantitative enumeration of *HER2* and Chr17 signals in 20 nuclei within a target area, and calculates the *HER2*/Chr17 ratio.

Utilizing stained serial slides that meet the criteria for Slide Adequacy, locate the same target area on each slide of *HER2* and Chr17 and count the number of signals in 20 cells located in the Target Area according to Table 2–Signal Enumeration. Calculate the *HER2*/Chr17 ratio by dividing the total number of *HER2* signals by the total number of Chr17 signals. Cases with a *HER2*/Chr17 ratio less than 1.8 are negative for *HER2* gene amplification, while cases with a *HER2*/Chr17 ratio greater than 2.2 are positive for *HER2* gene amplification and can be reported as such. Cases with a *HER2*/Chr17 ratio that is either equal to or falls between 1.8 and 2.2 must be enumerated using Method 2A to ensure accuracy (Figure 10).

Figure 10. Method 2 Scoring Algorithm



Method 2A: Additional Quantitative

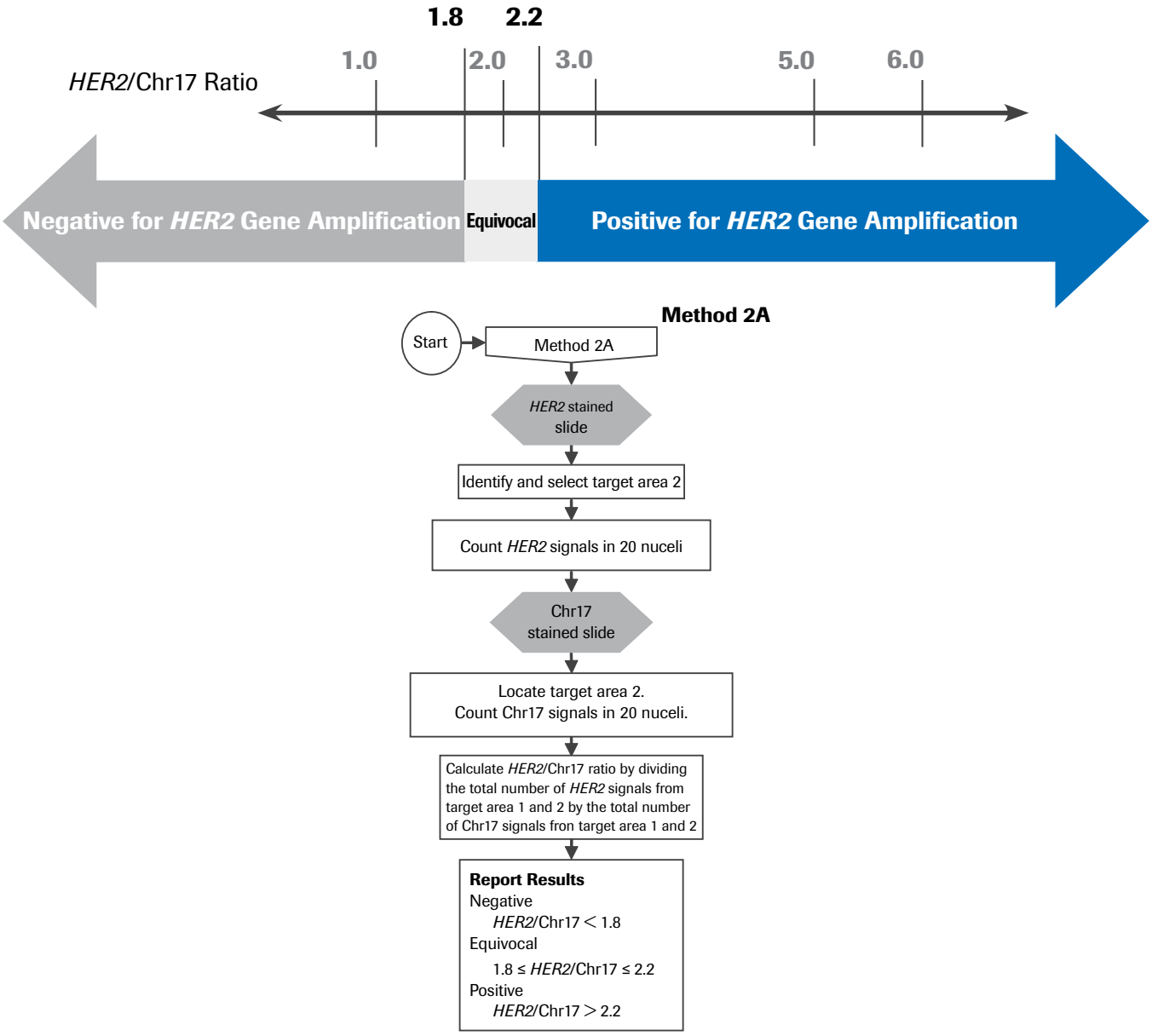
For cases that have been enumerated using Method 2 and have a *HER2*/Chr17 ratio either equal to or between 1.8 and 2.2, Method 2A must be utilized. The reader selects a second target area for each of *HER2* and Chr17 and counts the number of signals in 20 additional nuclei. The *HER2*/Chr17 ratio using counts from both target areas is then calculated. A total of 40 nuclei will have been read between both target areas. Calculate the *HER2*/Chr17 ratio by dividing the total number of *HER2* signals in both target areas (40 cells) by the total number of Chr17 signals in both target areas (40 cells). Results are reported as:

a. Negative for *HER2* gene amplification. Defined as a *HER2*/Chr17 ratio less than 1.8.

b. Equivocal for *HER2* gene amplification. Defined as a *HER2*/Chr17 ratio equal to or between 1.8 and 2.2.

c. Positive for *HER2* gene amplification. Defined as a *HER2* Chr17 ratio greater than 2.2.

Figure 11. Method 2A Scoring Algorithm



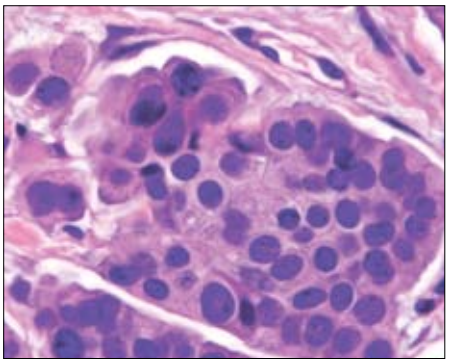
Examples of *HER2* and Chr17 Staining Patterns in Clinical Cases

The following images are provided to illustrate a variety of staining patterns that may be present in invasive breast carcinoma cases when stained with INFORM *HER2* DNA Probe: positive, negative, and equivocal for *HER2* gene amplification. The intended use of these photographs is to allow the new user of this test to become familiar with the spectrum of staining patterns they may encounter.

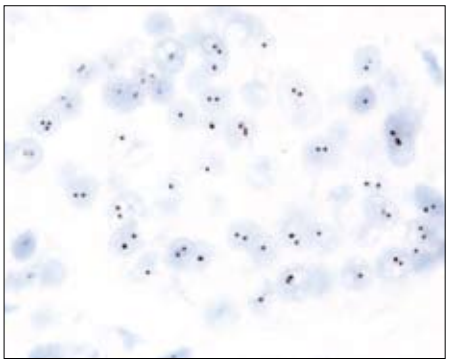
Any staining performed in the end user's laboratory should be interpreted within the context of the controls run with the clinical cases at the time of evaluation (See Quality Control for SISH Staining section).

- Photoset A provides an example of a case negative for *HER2* gene amplification, with matched Chr17 staining.
- Photoset B provides examples of cases positive for *HER2* gene amplification, cases 14 and 15. Case 16 shows a *HER2* amplified case with H&E and matched Chr17. Cases 17 and 18 provide examples of *HER2* amplified cases with emphasis on the presence of normal gene copy number in lymphocytes and fibroblasts adjacent to tumor.
- Photoset C provides an example of a case that was initially equivocal for *HER2* gene amplification, corrected to negative by polysomy Chr17 staining. This case illustrates the importance of a dual *HER2*/Chr17 result.

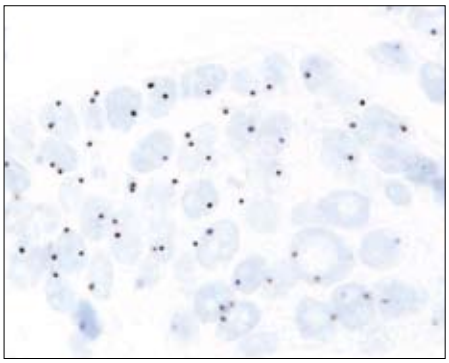
Photoset A. Case Negative for *HER2* Gene Amplification



Case 13: H&E 60X

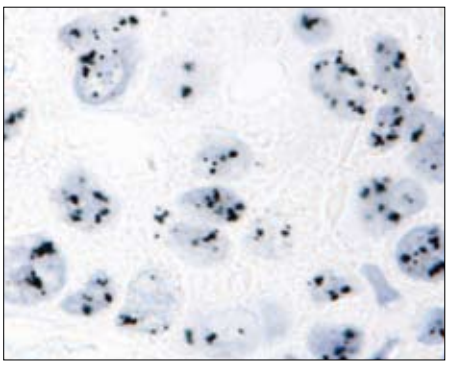


HER2 60X

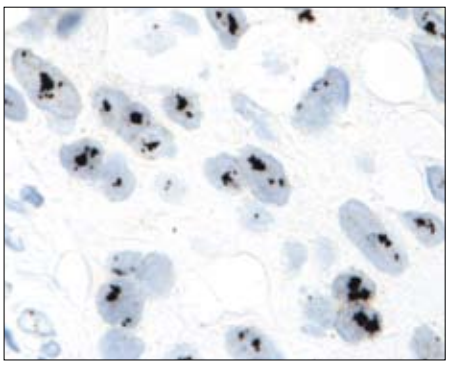


Chr17 60X

Photoset B. Cases Positive for *HER2* Gene Amplification

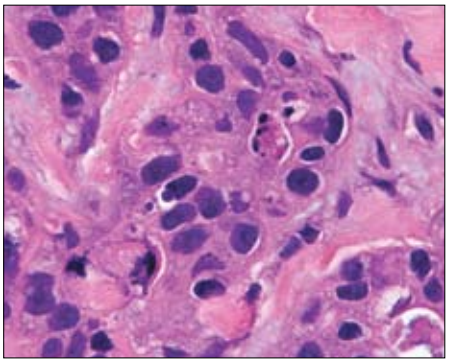


Case 14: *HER2* 60X

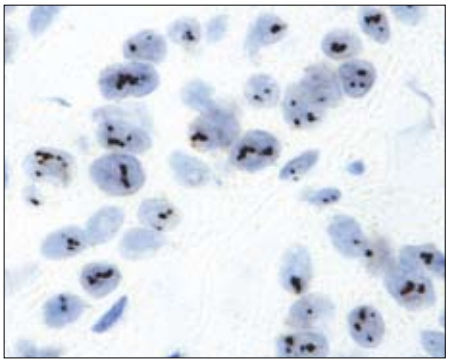


Case 15: *HER2* 60X

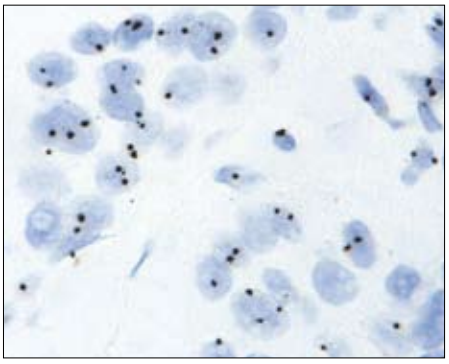
Photoset B. (cont) Cases Positive for *HER2* Gene Amplification



Case 16: H&E 60X

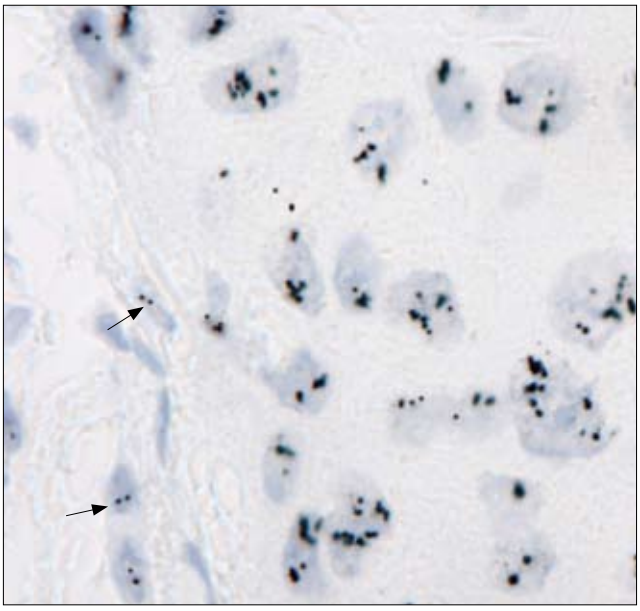


HER2 60X

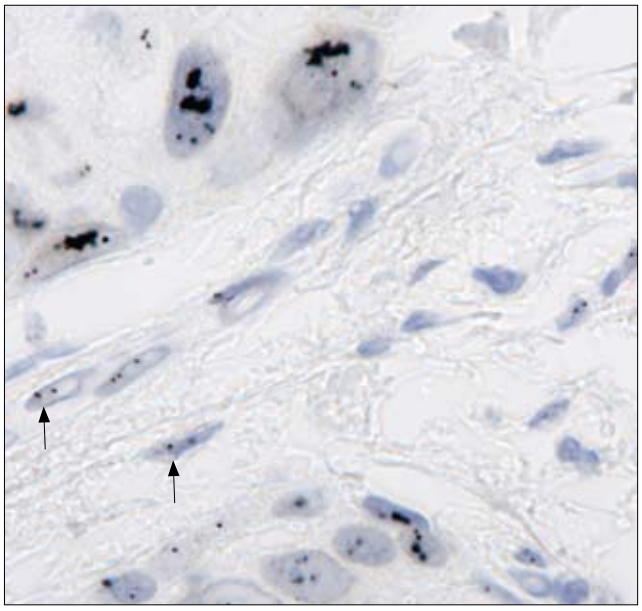


Chr17 60X

Note normal lymphocytes and fibroblasts (denoted with an arrow) adjacent to tumor.



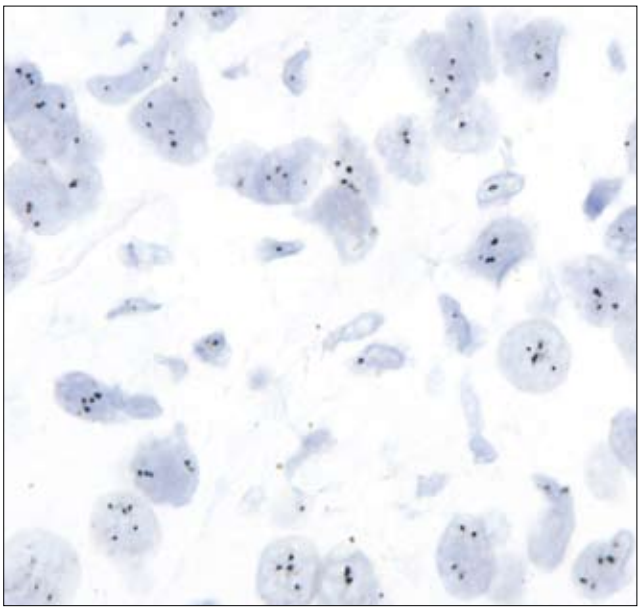
Case 17: *HER2* 60X



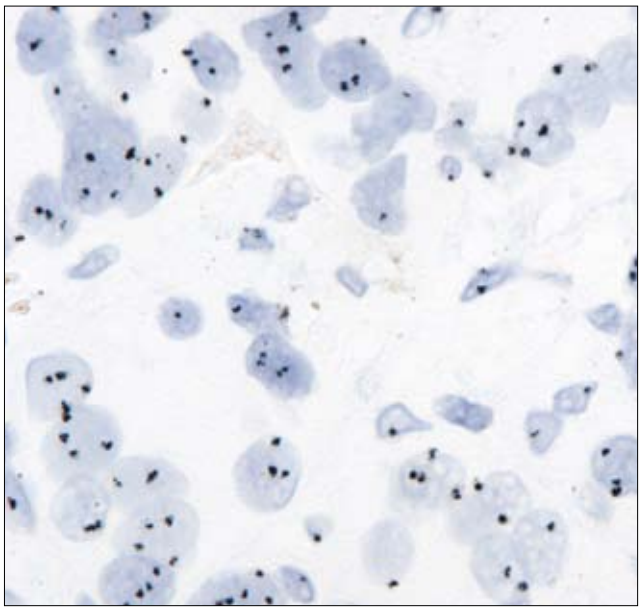
Case 18: *HER2* 60X

Photoset C. A Case Initially Equivocal for *HER2* Gene Amplification Corrected to Negative by Polysomy 17

Case 19 is an initially borderline *HER2* case corrected by polysomy 17 chromosome ratio to negative for *HER2* gene amplification.



Case 19: *HER2* 60X



Chr 17 60X

Quality Control For SISH Staining

Positive and Negative Controls for SISH Staining

Normal *HER2* signals (1 to 2 copies per cell) act as internal positive controls for each case and must be visible in the sample. Specific SISH nuclear staining may be located in various cells including: stromal fibroblasts, endothelial cells, lymphocytes, and non-neoplastic breast epithelial cells. However, not all cells will exhibit single gene copy due to biological heterogeneity and plane of sectioning. If normal HER2 signals are not evident, the slide is inadequate for interpretation.

A positive control may be included with every staining procedure performed. The controls are used to confirm that the reagents and the instrument have functioned properly. (It is important that the optimal staining conditions be established on biopsy control samples prior to running patient samples.)

Positive tissue controls should include biopsy specimens prepared in a manner identical to the patient specimens to be tested. These specimens should be used because they are known to have normal copies of *HER2* in both the control stromal cells and the invasive carcinoma cells. These serve as quality controls for all steps of the procedure, from the pre-analytical specimen preparation through the staining process. Use of a specimen prepared differently from the test specimens will provide control for all reagents and procedures except fixation and specimen processing.

Separate positive controls are not required.

Since the *HER2* gene and Chromosome 17 are present in every human cell, there is no true negative tissue control. For the purpose of a negative reagent control, Ventana produces an ISH negative control reagent that can be used in place of the DNA probes.

HER2 3-in-1 Xenograft Control Slides

Xenograft controls are useful for a preliminary validation of the processing methods used for staining clinical slides, and for use as controls on individual runs. Ventana offers the *HER2* 3-in-1 Xenograft Control Slides (p/n 783-4332), containing three distinct xenograft sections that are formalin-fixed and embedded in a single paraffin block (Figure 12). The cell lines (Calu-3 or BT474, ZR-75-1, and MCF-7), generated as xenograft sections, have been selected based upon their molecular characterization of *HER2* gene copy number and protein levels (from published literature), and represent the dynamic range of *HER2* copies observed in clinical samples (Tables 3 and 4). *Note that the xenografts contain host mouse cells interspersed with the human carcinoma cells. Human HER2 and Chr17 are not detectable in these mouse cells.*

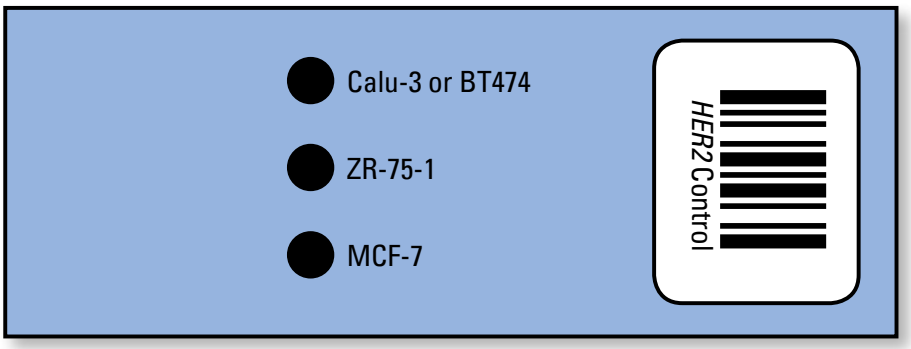
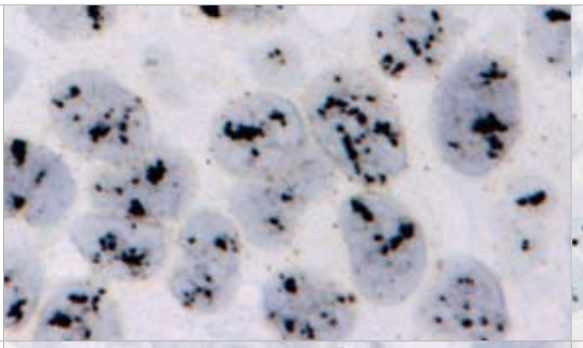
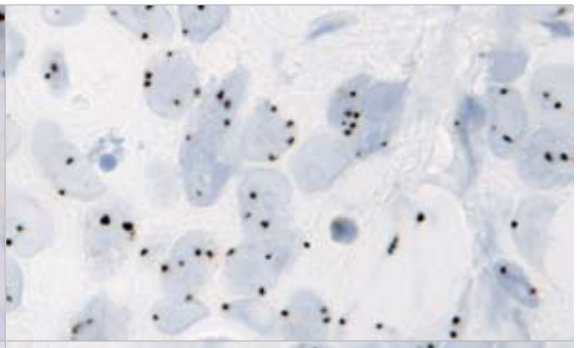
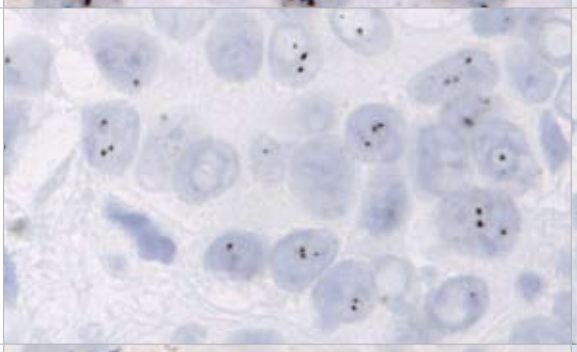
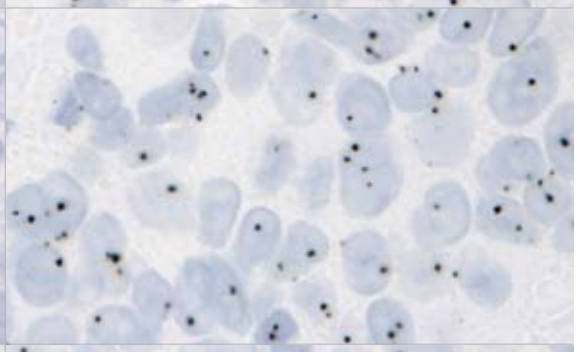
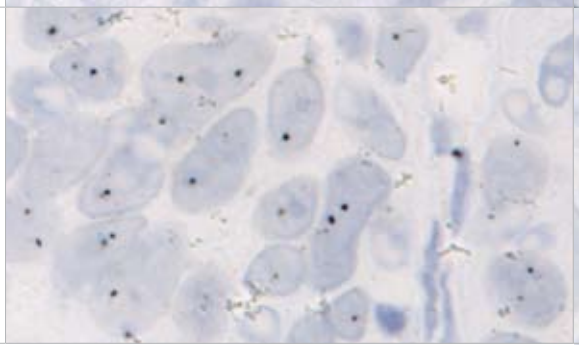
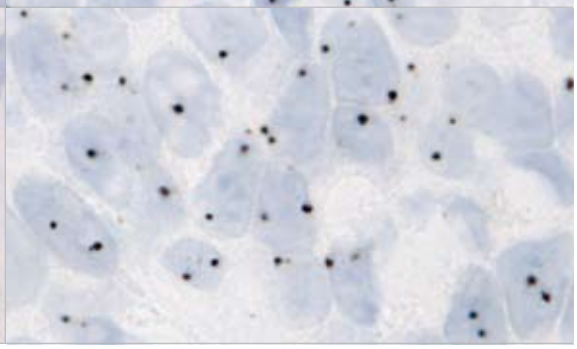


Figure 12. *HER2* 3-in-1 Xenograft Control Slides

Table 3. Characterization of *HER2* 3-in-1 Xenograft Control Slides

Cell Line	*HER2* Protein Expression Level	Approximate *HER2* Gene Copy# by FISH	Approximate Chr17 Copy# by FISH	*HER2* Gene Amplification
Calu-3 or BT474	3+	Equal to or greater than 20 copies/nuclei	3 copies/nuclei	Positive
ZR-75-1	1+	3 copies/nuclei	2 copies/nuclei	Negative
MCF-7	0	1-2 copies/nuclei	2 copies/nuclei	Negative

Table 4. Characteristic Staining Pattern of *HER2* 3-in-1 Xenograft Control Slides

Cell Line	INFORM™ *HER2* DNA Probe	INFORM Chromosome 17 Probe
Calu-3 or BT474		
ZR-75-1		
MCF-7		

Pre-Analytical Considerations and Optimization of the Assay

Pre-analytical sample preparation is an essential factor to consider before using any *in situ* hybridization assay. For the INFORM *HER2* DNA Probe, Ventana recommends that the tissue be fixed in 10% neutral buffered formalin (NBF) for 6-24 hours, paraffin embedded, and sectioned at approximately four microns. Ventana has developed the INFORM *HER2* DNA Probe assay with protease treatment options that will enable optimization of the assay in different laboratories, and for subsequent troubleshooting of individual slides that may exhibit sub-optimal staining. It is recommended that each laboratory perform staining runs on representative biopsy samples that have been prepared under the identical conditions as the clinical samples to be tested. This will aid in optimizing the specific SISH staining conditions for individual laboratories that may vary in their exact specimen preparation procedures.

Fixation

For samples fixed in 10% NBF and sectioned at approximately four microns, Ventana ISH Protease 3 is recommended for eight minutes, for both *HER2* and Chr17 probes. Tissue fixed in either 10% NBF (recommended) or Zinc formalin are suitable specimen types for the INFORM HER2 DNA Probe Assay. Tissue fixed in alcoholic formalin may be a suitable specimen type but may not stain as robustly as tissue fixed in either 10% NBF or Zinc formalin. **Prefer and Bouin's fixatives were demonstrated to be incompatible with this assay, as samples fixed using these two fixatives failed to yield single copy gene detection**. Tissues that have been fixed inappropriately sometimes can be identified by the presence of peripheral SISH staining, with weak or absent staining in the center of the tissue. Troubleshooting for weak or absent staining, as discussed in the Troubleshooting Section, is recommended. This involves treatment of the tissue with a stronger protease (e.g., ISH Protease 2 for four minutes or ISH Protease 3 for 12-24 minutes). For additional information on fixation for ISH, refer to the CAP/ASCO Guideline Recommendations for HER2 Testing.[22]

Sample Thickness

Samples with a thickness of greater than four microns likely will require the use of a stronger protease treatment, such as Ventana's ISH Protease 2 for a treatment time of four minutes or longer. This protease treatment is effective at unmasking DNA in thicker samples. Thinner sections may require gentler protease treatments (i.e., ISH Protease 3 for four minutes) if excessive nuclear background staining is observed (See Troubleshooting).

Other Considerations

Type of specimen

It also is important to consider the type of specimen to be tested in the assay (for example, tissue obtained from a needle biopsy vs. an excisional biopsy). Optimizing the ISH Protease treatment conditions on each specimen type may be necessary if differences in staining quality are observed.

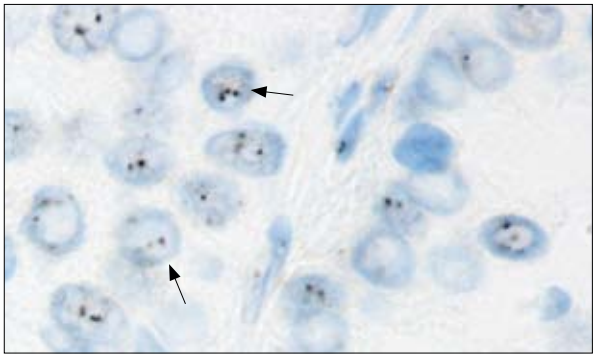
Overall sample quality

SISH staining may be sub-optimal in tissue containing areas of crush artifact, necrosis, or in tissue of overall poor morphology. It is recommended that H&E staining be evaluated from each case to evaluate overall specimen morphology.

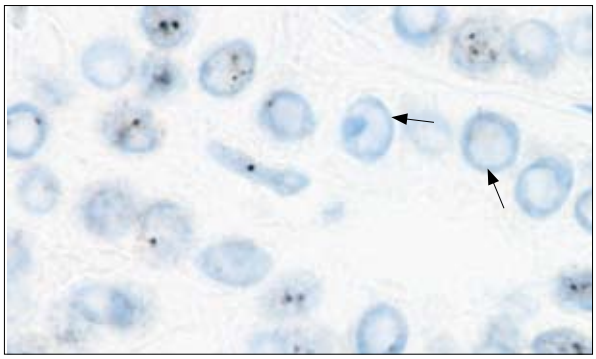
The balance between nuclear morphology and SISH signal intensity.

In specimens prepared differently from the recommended procedures, it often is possible to visualize adequate SISH staining by optimizing the ISH Protease treatment. However, the use of ISH Protease 2 or 3 for extended amounts of time may affect the nuclear morphology and nuclear counterstain. Each reader should be aware of this and balance SISH signal intensity with nuclear morphology and counterstain. Furthermore, *HER2* or Chr17 SISH staining may decrease in intensity if too harsh a Protease treatment is used. (For samples that exhibit a weak counterstain, increasing the Hematoxylin II staining time, and the Bluing Reagent staining time, from the recommended four minutes to eight minutes may enhance the intensity of the counterstain).

Tissue that has been pre-analytically processed in a suboptimal manner may exhibit “nuclear bubbling”. In many cases, the nuclear bubbling does not interfere with the SISH staining such as in Case 20, and cells may be enumerated if SISH signal is adequate. However, if bubbling precludes the SISH signal, affected cells should not be scored (Case 21).



Case 20



Case 21

Troubleshooting

This section addresses issues that may be encountered with the assay.

Lack of Staining on Xenograft Control Slides

If there is a lack of expected staining of the *HER2* 3-in-1 Xenograft Control Slides (i.e., an absence of single copy staining of *HER2* or Chromosome 17), check to ensure that all dispensers are functioning properly.

Weak or Absent Staining

When cases (both *HER2* and Chr17) exhibit weak or absent staining (and the weak staining is found to interfere with cell signal enumeration), this often indicates a problem with specimen preparation. Repeat staining on the case using a stronger protease treatment (e.g., ISH Protease 2 for four minutes) or a hybridization time longer than six hours. If a case has stained appropriately for one probe but not for the other (e.g., *HER2* stains appropriately but Chr17 exhibits absent staining), repeat the staining of the affected slide utilizing the same protocol. If the staining is still inadequate, this often indicates a problem with specimen preparation and staining should be repeated utilizing a stronger protease treatment or a longer hybridization time.

Weak or Absent Counterstain

For cases that exhibit a nuclear counterstain that is weak or absent (making it difficult to discern nuclear boundaries), increase the counterstaining conditions from 4 minutes to 8 minutes. Weak counterstaining can indicate that the specimen has not been prepared as recommended. Also, see the Pre-Analytical Considerations section for more information on the balance between nuclear morphology and SISH signal intensity.

Non-Specific Staining—Slide Drying Artifact

If a case or xenograft control slide exhibits uninterpretable SISH staining, with brown or black background throughout the tissue, the slide has dried and must be re-run. If excess silver is deposited on the slide, making it difficult to enumerate the nuclear SISH signal, the slide must be re-run. Check that the instrument and reagent dispensers are functioning properly, and that the bulk reagents are adequately filled. Also check to make sure that the dispensers have not been stored without their caps on, as silver acetate is known to oxidize over time.

Non-Specific Staining—Nuclear Dust or Haze

If the slides exhibit excessive background staining that interferes with enumeration of the specific SISH signals (i.e., nuclear dust or haze), the use of a gentler protease treatment (e.g., ISH Protease 3 for four minutes, instead of 8 minutes), is recommended.

Weak or Absent Staining of Positive Control

If the positive control is negative or exhibits weaker staining than expected, other positive controls stained on the same staining run should be checked to determine whether the failure is due to the control slide or the reagents used. Ventana's *HER2* 3-in-1 Xenograft Control Slides serve as run controls to ensure that the reagents and instrument are functioning properly.

Oxidizing or Fading of SISH Signal

Oxidation, fading, and/or disappearance of the SISH signal may be due to certain brands of mounting media. **The following mounting media are known to cause oxidation: EUKITT (EMS), Entellan (Merck), and Entellan NEW (Merck).** Please refer to Table 5 for mounting media compatibility. If the mounting media you are using is not included in this list, we recommend that you validate that the media is compatible with the test system prior to use with patient samples. Internal studies have shown that fading usually occurs within a few hours to a week.

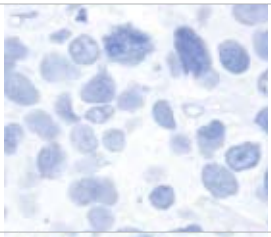
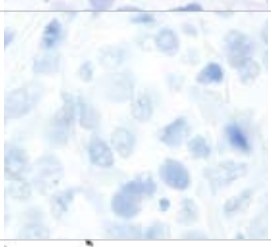
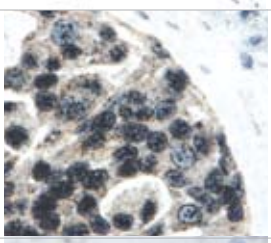
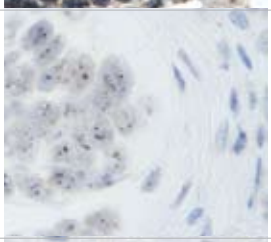
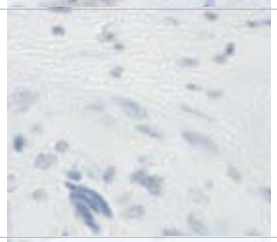
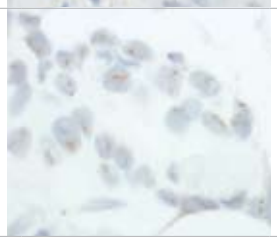
As further guidance, Table 6 has been created to help troubleshoot some of the issues described above.

For additional information, refer to the Step-By-Step Procedure section in the automated slide stainer Operator's Manual or contact your local Ventana office.

Table 5. Mounting Media Compatibility

Mounting Media	Manufacturer	Compatible with SISH?
Eukitt	EMS	No
Entellan New	Merck	No
Entellan	Merck	No
Cytoseal XYL	Richard Allan Scientific	Yes
Paramount	Protaqs Quartett: Dako	Yes
DPX	BDH: Raymond Lamb	Yes
Cytoseal 60	Richard Allan Scientific	Yes
Permount	Fisher	Yes
Histomount	Raymond Lamb	Yes
Ultramount	Dako	Yes
Thermo EZ Mount	Thermo Scientific	Yes
SureMount	Triangle Biomedical Sciences	Yes
Flo-Texx	Lerner Labs	Yes
Mountex	Histolab	Yes
Shandon Consul mount	Thermo Scientific	Yes
MM24	SurgiPath	Yes
Pertex	Cell Path	Yes
MicroMount	SurgiPath	Yes
Diamount	Diapath	Yes
Alcolmount	Diapath	Yes
BioMount 2	BB International	Yes
Acrytol	SurgiPath	Yes
Gel Mount	Biomeda	Yes
Mount-Quick	Daido Sangyo Co.	Yes

Table 6. Troubleshooting

Lack of staining on xenograft control slides		If there is a lack of expected staining in the *HER2* 3-in-1 Xenograft Control Slides (i.e., an absence of single copy staining of *HER2* or Chromosome 17), the run is invalid. Check to ensure that all dispensers are functioning properly.
Weak or absent staining		Determine if weak staining interferes with slide enumeration. If staining is weak or absent, use a stronger protease treatment and/or increase the hybridization time to greater than 6 hours.
Weak or absent counterstain		Determine if weak counterstaining interferes with slide enumeration. If weak counterstaining interferes with slide enumeration, then repeat the staining of the affected slide(s) and either increase the counterstain time or utilize a gentler protease treatment (i.e., ISH Protease 3 for 4 min.). For more information regarding the balance between nuclear morphology and SISH signal intensity, review the section on Pre-Analytical Considerations and Optimization of the assay.
Non-specific staining: slide drying artifact		Check to make sure all bulk fluids are filled and reagent dispensers are working properly. Repeat the staining of the affected slide(s) using the same protocol. If staining is still unacceptable, follow instrument user's manual troubleshooting.
Non-specific staining: nuclear dust or haze		Determine whether non-specific staining interferes with slide eneumeration. If non-specific staining interferes with slide enumeration, then repeat the staining of the affected slide with a gentler protease treatment (i.e., ISH Protease 3 for 4 min.) and/or reduce hybridization time to less than 6 hours.
Weak or absent staining of positive control		If the positive control is negative or exhibits weaker staining than expected, other positive controls stained on the same staining run should be checked to determine whether the failure is due to the control slide or the reagents used. Ventana's *HER2* 3-in-1 Xenograft Control Slides serve as run controls to ensure that the reagents and instrument are functioning properly.
Oxidizing or fading of SISH signal		This may be due to certain brands of mounting media. If the SISH signal of the sample begins to oxidize (turning orange) or completely disappears, restain the case and coverslip using a different mounting media.

If a condition persists across multiple cases and repeated attempts to correct the condition, contact your local Ventana office.

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Appendix A: Score Sheets

Interpretation of Results – Score Sheet for Method 1

Method #1

HER2: Estimated average number of *HER2* Signals in Target Area 1	a
Chr17: Estimated average number of Chr17 Signals in Target Area 1	b
***HER2*/Chr17 Ratio**	c = a/b
☐ **Negative: *HER2*/Chr17 < 1.4**	
☐ **Positive: *HER2*/Chr17 > 4.0**	
☐ **1.4 ≤ *HER2*/Chr17 ≤ 4.0 Use Method #2**	

Interpretation of Results – Score Sheet for Method 2 and Method 2A

Method #2

Target Area 1

Cell	*HER2* Count	Cell	Chr17 Count
1		1	
2		2	
3		3	
4		4	
5		5	
6		6	
7		7	
8		8	
9		9	
10		10	
11		11	
12		12	
13		13	
14		14	
15		15	
16		16	
17		17	
18		18	
19		19	
20		20	
Total number of *HER2* signals in Target Area 1		Total number of Chr17 signals in Target Area 1	
d		e	
Target Area 1 *HER2*/Chr17 Ratio			
f = d/e			
☐ Negative: *HER2*/Chr17 < 1.8			
☐ Positive: *HER2*/Chr17 > 2.2			
☐ 1.8 ≤ *HER2*/Chr17 ≤ 2.2 Use Method #2A			

Target Area 2

Cell	*HER2* Count	Cell	Chr17 Count
1		1	
2		2	
3		3	
4		4	
5		5	
6		6	
7		7	
8		8	
9		9	
10		10	
11		11	
12		12	
13		13	
14		14	
15		15	
16		16	
17		17	
18		18	
19		19	
20		20	
Total number of *HER2* signals in Target Area 2		Total number of Chr17 signals in Target Area 2	
g		h	
Target Areas 1 and 2 *HER2*/Chr17 Ratio			
k = (d+g)/(e+h)			
☐ Negative: *HER2*/Chr17 <1.8			
☐ Positive: *HER2*/Chr17 > 2.2			
☐ Equivocal: 1.8 ≤ *HER2*/Chr17 ≤ 2.2			

Patient Centric Pathology

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